Role of T Lymphocytes in Liver Abscess Formation by *Bacteroides fragilis* in Mice

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The underlying mechanisms of liver abscess formation have not been fully elucidated with regard to the interaction between bacterial virulence factors and the immune response. The objective of this study was to determine the role of the host T cells in liver abscess formation caused by *Bacteroides fragilis*. We developed a liver abscess mouse model with inoculation of *B. fragilis* through the hepatic portal vein and examined the role of T cells by studying T cell-deficient mice, as well as conducting adoptive T cell transfer experiments. No microabscess was formed in the αβ T cell receptor-positive (αβTCR++) T cell-depleted mice, in contrast to the results for the control mice. In addition, the αβTCR knockout (KO) mice showed significantly lower numbers of microabscesses, and the abscesses were smaller in size than those in the wild-type mice. Adoptive transfer of T cells purified from the wild-type mice into the αβTCR KO mice resulted in liver abscess formation in those mice. These findings suggest that T cells play an essential role in liver abscess formation caused by *B. fragilis* in mice.

The incidence of pyogenic liver abscess has remained relatively constant during this century (16). The major organisms causing pyogenic liver abscess are aerobic Gram-negative Enterobacteriaceae, including *Escherichia coli*, *Klebsiella pneumoniae*, and anaerobic bacteria, especially *Bacteroides fragilis* (9). *B. fragilis* has been recognized as one of the most important organisms causing pyogenic liver abscess, usually along with other enteric pathogens (3, 16). In general, infection is initiated after bacterial entry into the liver through the hepatic portal vein from lesions at a site drained by the portal vein or through the biliary tract from the intestinal lumen (11, 19). The pathological findings of abscess formation are characterized by infiltration of neutrophils and deposition of fibrin, resulting in a pus-filled cavity with capsule formation. The formation of an abscess is thought to result from the response of the host defense mechanisms that act to prevent dissemination of the bacterial infection.

Active research on the pathogenesis of intraperitoneal abscess formation has documented the interaction between the virulence factors of *B. fragilis* and host T lymphocytes (20–22). *B. fragilis* is the most important anaerobe that causes intraperitoneal infections (15), although it accounts for less than 0.5% of the microorganisms resident in the human colon (7). Capsular polysaccharides of *B. fragilis*, which have a distinct zwitterionic structure, are known to be essential virulence factors required for intraperitoneal abscess formation by this organism. T cell immune responses, directly activated by these polysaccharides, play an important role in the development of intraperitoneal abscess (20–22). That is, intraperitoneal abscess formation is regulated by the immune response mediated by T cells that are activated by bacterial polysaccharides.

Thus, the interaction between the polysaccharides of *B. fragilis* and the host T cell responses plays an essential role in the development of intraperitoneal abscess formation (20). However, there are no data on the development of liver abscesses with regard to the role of T cells and their interaction with *B. fragilis*. The liver is a reticuloendothelial organ that is critical in the host defense system against bacterial invasion. From an immunological point of view, the liver is a unique organ in the peritoneal cavity. Kupffer cells are resident tissue macrophages found in the sinusoids of the periportal area; they are the first macrophages encountered by the microorganisms or microbial products transported from the intestines to the liver (2). Their function of rapid clearance of bacteria is thought to be due to a complex interaction between the Kupffer cells and immigrant neutrophils (8). In order to demonstrate the mechanism involved in the development of pyogenic liver abscesses in response to *B. fragilis*, independent investigations utilizing animal models of liver abscess formation are required.

The purpose of this study was to investigate the role of T cells in liver abscess formation caused by *B. fragilis*. We developed a mouse model of liver abscess by inoculating *B. fragilis* through the hepatic portal vein and examined the role of T cells by immunological methods.

MATERIALS AND METHODS

Animals. C57BL/6 mice (4 to 6 weeks, male) were obtained from Orient Bio, Inc. (South Korea). The αβ T cell receptor knockout (KO) mice (αβTCR−/−); B6.129P2-Tcrb and wild-type mice were obtained from the Jackson Laboratory (ME). All animals were provided with food and water *ad libitum*. The animal...
experiments were performed in accordance with the guidelines established by the Hallym University College of Medicine Standing Committee on Animals.

Preparation of B. fragilis inoculums. B. fragilis NCTC 9343, successfully used for the development of an intraperitoneal abscess model (22), was used for bacterial inoculums to induce liver abscess formation in the mice. Stock bacteria were inoculated onto brucella blood agar plates and cultured at 37°C over 24 h in an anaerobic jar (GasPak system; BBL, United States). After subculture in thiglycolate broth for 18 h, diluted broth was used to inoculate fresh thiglycolate broth. The bacterial suspension was obtained at the exponential phase of growth. We prepared B. fragilis inoculums with doses of 2 × 10^7 CFU/mouse in a volume of 0.1 ml phosphate-buffered saline (PBS).

Mouse model of pyogenic liver abscess formation. The mice were anesthetized with a single intraperitoneal injection of 0.15 ml of 1:5 (vol/vol)-diluted pentobarbital sodium solution (10 mg/ml; Hanlim Pharm. Co. Ltd., South Korea). After shaving and disinfection with povidone iodine solution, an anterior midline incision was made through the abdominal wall and peritoneum. The intestines were carefully pulled out and laid laterally over a sterile drape, and then the hepatic portal vein was identified. The bacterial inoculum prepared in a 0.1-ml volume was injected into the hepatic portal vein using a 30-gauge insulin syringe. For hemostasis, following removal of the needle, the vein was lightly compressed using sterile gauze. The abdominal wall was closed with two layers of interrupted sutures. Some animals that had massive bleeding or died during this procedure and those who died within 12 h after the procedure were excluded from the outcome analysis. The animals were sacrificed using CO₂ euthanasia and examined for liver abscess formation 21 days later.

Examination for B. fragilis-induced liver abscess formation in mice. In order to study liver abscess formation caused by B. fragilis, small liver sections were cultured under anaerobic conditions. Isolated bacterial colonies were Gram stained, and anaerobic identification was performed with a RapID ANA II kit (Remel, Inc., Lenexa, KS). The presence of a liver abscess was histopathologically confirmed. The liver was fixed in 4% paraformaldehyde and embedded in paraffin. Histological sections were stained with hematoxylin and eosin (H&E). The serial sections included all of the hepatic lobes that were examined for histological evidence of liver abscess formation. To exclude simple neutrophilic infiltrations from our analysis, a microabscess was defined as a focal infiltration of neutrophils that was 50 μm or larger in diameter and that was accompanied by the destruction of a normal hepatic parenchyma. The number of microabscesses observed in 100 microscopic fields at a magnification of ×100 was determined for each mouse. The maximal diameter of the microabscesses was also determined. The median numbers of microabscesses and the maximal diameters were compared among the study groups. The microabscesses were measured using an ocular micrometer (Nikon Instruments).

Induction of liver abscess formation in the αβTCR⁻ T cell-depleted mice. T cells bearing αβTCR in the C57BL/6 mice were depleted with the treatment of 300 μg TCR β chain-specific monoclonal antibody (MAB) H57-597 (BD Pharmingen, United States) via the intraperitoneal route 4 days before surgery (6). The control mice were treated with the same amount of isotype-matched antibody. The bacterial inoculum was injected through the portal vein, and the development of hepatic microabscesses was assessed as described above.

Depletion of the αβTCR⁻ T cells was confirmed by fluorescence-activated cell sorting (FACS) analysis 4 days after treatment with antibody. Peripheral blood mononuclear cells were purified from the mice injected with MAB H57-597, as well as from those receiving control antibodies, and then tested for the proportion of αβTCR⁻ T cells. The cells were stained with FITC (fluorescein isothiocyanate)-labeled or PE (phycoerythrin)-labeled MABs to CD3 and TCRβ and isotype control antibodies (BD Pharmingen, United States). The stained cells were analyzed on an BD FACSCalibur (BD Biosciences, United States) using CellQuest software (BD Biosciences, United States) and WinMDI 2.8 analysis software (http://facs.scripps.edu; Scripps Research Institute).

Induction of liver abscess formation in αβTCR⁻ mice. The αβTCR⁻ mice and wild-type mice were injected with B. fragilis inoculums through the hepatic portal vein and were assessed for the development of liver abscess formation as described above.

Adaptive T cell transfer. Splenic mononuclear cells from the wild-type mice were separated by centrifugation with Ficoll-Hypaque gradient (Lymphoprep; Axis-Shield, Norway), and the T cells were purified on a nylon wool column (Polysciences, Warrington, PA). T cell-enriched populations (>90% T cells) were transferred to the αβTCR⁻ mice (2 × 10^7 cells/mouse) by the intracardiac route 24 h before inoculation with B. fragilis. The control group received intracardiac injection of PBS instead of purified T cells. As another control, wild-type mice were inoculated with B. fragilis inoculums without T cell transfer.

Statistical analyses. Evaluation of the differences in the numbers and maximal diameters of the microabscesses was performed with the Mann-Whitney U test (SPSS version 11.0; SPSS Inc., Chicago, IL). A P value of < 0.05 was considered to be statistically significant.

RESULTS

Development of B. fragilis-induced liver abscess model in mice. To demonstrate the liver abscess formation caused by B. fragilis, small sections of the livers from the mice were studied on day 21 after inoculation. The section was cultured anaerobically in thiglycolate broth in an anaerobic jar at 37°C over 48 h. When diluted broth was subcultured into the brucella blood agar, small, white, and even colonies were observed (Fig. 1A). The Gram stain revealed pleomorphic Gram-negative organisms (Fig. 1B). The disk diffusion test showed resistance to vancomycin, kanamycin, and colistin on the brucella blood agar plates. Culture on bacteroides bile esculin agar showed black colonies and black pigmentation of the agar that was identified as B. fragilis by the RapID ANA II system (Remel Inc., Lenexa, KS). Histopathological examination of the liver sections revealed many microabscesses with various diameters (50 to 250 μm) (Fig. 1C and D). Most of the infiltrating cells were polymorphonuclear leukocytes.

Induction of liver abscess formation in T cell-depleted mice. Flow cytometry analysis showed that H57-597, a MAB specific for the αβTCR β chain, successfully depleted αβTCR⁻ T cells in the mice (Fig. 2A). All eight mice that were given control antibody developed microabscesses with diameters greater than 50 μm 21 days after inoculation with B. fragilis (Fig. 2B and D). The median number of microabscesses with diameters greater than 50 μm in 100 microscopic fields at a magnification of ×100 was 10 (range, ~7 to 18) in mice given the control antibody (Fig. 2B). The median for the maximal diameter of the microabscesses was 150 μm (range, ~80 to 220) (Fig. 2C). In contrast, none of the mice depleted of αβTCR⁻ T cells developed microabscesses (Fig. 2E).

Induction of liver abscess formation in αβTCR KO mice. To further demonstrate the role of αβTCR⁺ T cells in the development of liver abscess formation caused by B. fragilis, αβTCR⁻/⁻ mice were studied. Among the 10 αβTCR⁻/⁻ mice injected with B. fragilis inoculums, one died 2 days later and three died 3 to 4 days later; these mice were excluded from the analysis. Only two out of six TCR KO mice developed microabscesses, in contrast to the 12 wild-type mice, which all developed hepatic microabscesses with diameters greater than 50 μm. The median number of microabscesses in the wild-type mice was 11 (range, ~5 to 32), and the median for the maximal diameter was 150 μm (range, ~90 to 250). In contrast, the numbers of microabscesses found in the αβTCR⁺/⁻ mice were 1 and 2, respectively, and the maximal diameters were 70 μm and 90 μm (Fig. 3A and B).

αβTCR⁺ T cells are critical for the liver abscess formation caused by B. fragilis. To further demonstrate the role of the TCR⁺ T cells in the development of liver abscess formation caused by B. fragilis, we performed T cell transfer experiments in which the αβTCR⁻/⁻ mice, previously shown to be genetically impaired in their ability to develop liver abscesses, were used as recipients. A total of 2 × 10⁶ T cells was transferred into each mouse, and flow cytometric analysis showed that 81% of the cells were CD3⁺ T lymphocytes. Among eight αβTCR⁻/⁻ mice that received intracardiac injection of phos-
FIG. 1. Demonstration of liver abscess formation in mice injected with *B. fragilis* inoculums. (A) Bacterial colonies isolated in brucella blood agar plates under anaerobic conditions. Small, white, and even colonies are observed. (B) Gram stain revealed pleomorphic Gram-negative organisms. (C) Histopathological evidence of microabscess on day 7 (magnification, ×100, inset ×400; H&E stain). Infiltration of polymorphonuclear leukocytes is seen along with destruction of normal hepatic parenchyma. (D) Microabscess formation on day 21 (magnification, ×100, inset ×400). The results shown are representative of two independent experiments.
phate-buffered saline, three died 3 to 4 days after inoculation with *B. fragilis*. In contrast, two of five *H9251/H9252 TCR* mice given purified T cells died 7 and 9 days, respectively, after *B. fragilis* inoculation.

The numbers of microabscesses in the five surviving *H9251/H9252 TCR* mice receiving saline instead of T cells as a control were significantly less than the numbers in the wild-type mice (*P* = 0.002) (Fig. 4A). However, the numbers of microabscesses in the *H9251/H9252 TCR* mice after adoptive transfer of the T lymphocytes were not significantly different from the numbers in the wild-type mice (Fig. 4A). The maximal diameters of the microabscesses did not differ between the *H9251/H9252 TCR* mice that received adoptive transfer of T lymphocytes and the wild-type mice; this was in contrast to the very small diameters found in the *H9251/H9252 TCR* mice that received saline transfer (Fig. 4B).

**DISCUSSION**

The results of this study show that T lymphocytes play an essential role in the development of liver abscess caused by *B. fragilis*. To demonstrate the role of T lymphocytes, we devel-

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**FIG. 2.** Loss of liver abscess-forming capability in mice depleted of αβTCR−/− T cells. (A) Depletion of αβTCR−/− CD3+ T cells in mice injected with the monoclonal antibody H57-597. (B) Comparison of the numbers of hepatic microabscesses in mice injected with the H57-597 monoclonal antibody and control antibody (*, *P* = 0.003, Mann-Whitney U test). (C) Comparison of the maximum diameters of the microabscesses in the two groups, (**, *P* = 0.003, Mann-Whitney U test). The horizontal bars indicate median values. (D) Microabscesses induced by *B. fragilis* in control antibody-treated mice (magnification, ×100; H&E stain). (E) No microabscesses were found in the H57-597-treated mice (magnification, ×100). The results shown are representative of two independent experiments.
oped a mouse model of *B. fragilis*-induced liver abscess formation and studied the mice with a T cell deficiency. Intraperitoneal injection of H57-597, a monoclonal antibody specific for the /H9251/H9252 TCR /H9252 chain, removed the /H9251/H9252 TCR /H11001 T cells effectively, as shown by the results of the flow cytometric analysis. These /H9251/H9252 TCR /H11001 T cell-deficient mice were impaired in microabscess formation, in contrast to the mice that received the control antibodies. The role of T lymphocytes in the liver abscess formation was further tested using knockout mice. The /H9251/H9252 TCR KO mice developed fewer microabscesses, and their microabscesses were much smaller than those in the wild-type mice. The finding that adoptive transfer of T cells enabled these KO mice to fully recover the capability of abscess formation clearly showed that T lymphocytes are essential for the liver abscess formation by *B. fragilis*. In addition, the finding that some of the /H9251/H9252 TCR KO mice showed a few microabscesses suggests that γδ TCR T cells might also have some role. There is also the possibility that NK T cells, which express αβ TCR, might be involved in the development of liver abscesses.

Previously reported animal models of pyogenic liver abscess formation most commonly used injection of anaerobic bacterial inoculums through the mesenteric vein in rabbits or the hepatic portal vein in rats or intraperitoneal injection in mice (1, 10, 14, 18). We chose mice for our study because monoclonal antibodies targeting surface antigens of T lymphocytes were broadly available and knockout mice could also be studied. We injected the *B. fragilis* inoculum into the hepatic portal vein to mimic the common natural route of transmission in the development of human liver abscess.

When the mice died during the inoculation through the hepatic portal vein procedure or immediately after surgery, they were excluded from the analysis because the mortality was considered to be related to noninfectious causes, such as bleeding, overdose of anesthesia, or hypothermia. Excluding these animals, the wild-type mice generally survived until day 21, when they were sacrificed. This low mortality in mice infected with *B. fragilis* can be explained by the low endotoxin activity of the lipopolysaccharides of *B. fragilis*, which seldom induce disseminated intravascular coagulation and severe sepsis (12). In part, successful localization of the bacterial infection to the liver, with effective killing and abscess formation, might have contributed to protecting the mice from high-grade bacteremia and mortality. In contrast, a few of the mice with a T cell deficiency died several days after inoculation with *B. fragilis*. This might have been due to their poor immune response to the bacterial invasion of the liver, as suggested by the inability to develop liver abscesses in the mice that survived.

FIG. 3. Loss of liver abscess-forming capability in the αβ TCR knockout (KO) mice. (A) Comparison of the numbers of hepatic microabscesses in the αβ TCR KO mice and the wild-type mice (*, *P* = 0.001, Mann-Whitney U test). (B) Comparison of the maximum diameters of the microabscesses in the two groups (**, *P* = 0.001, Mann-Whitney U test). The horizontal bars indicate median values. (C) Microabscesses induced by *B. fragilis* in the wild-type mice (magnification, ×100, H&E stain). (D) Few microabscesses were found in the αβ TCR KO mice (magnification, ×100). The results shown are representative of two independent experiments.
It is not clear how T cells specifically contribute to the liver abscess formation caused by \textit{B. fragilis}. The alleged mechanism involved in intraperitoneal abscess formation caused by \textit{B. fragilis} might partially explain it. The activation of CD4$^+$ T cells by the zwitterionic polysaccharide antigens is one of the critical factors involved in the complex host response to bacterial infection that results in abscess formation (5, 21). The subsequent release of cytokines from activated T cells stimulates the entry of neutrophils into the infection site. Recently, this process has been found to depend on the initial interaction of the zwitterionic polysaccharides with Toll-like receptor 2 (TLR2) (23). Based on the findings in murine models of intraperitoneal abscess formation, it is likely that zwitterionic capsular polysaccharides of \textit{B. fragilis} activate T cells and that the cytokines secreted by these T cells play an important role in the recruitment of neutrophils into the liver that are the predom-
inflammatory cells in abscess formation. Further experiments using knockout mice defective in production of the capsular polysaccharides will be necessary to demonstrate the role of the capsular polysaccharides in T cell-mediated liver abscess formation by \textit{B. fragilis}.

The innate immunity of the liver against bacterial invasion is unique and complex. The anatomical structure of the liver enables maximal contact of circulating blood with hepatocytes, which aids the host’s defense (2). Kupffer cells, located at the luminal surface of the endothelial cells, become activated in response to bacterial invasion and produce proinflammatory cytokines and chemokines (4, 13, 17). However, the resident immune responses in the liver are not limited to Kupffer cells. Additional cells include natural killer cells, CD4$^+$ T cells, and CD8$^+$ T cells. Furthermore, monocytes and neutrophils that circulate through the rich blood supply in the liver participate in the hepatic immune system (2). Therefore, the role of T cells in the liver abscess formation caused by \textit{B. fragilis} is part of the complex interaction between the host’s immune system and bacterial virulence. The possible mechanisms involved in the liver abscess formation caused by \textit{B. fragilis} include activation of Kupffer cells following the invasion of \textit{B. fragilis} into the liver, followed by the release of cytokines and chemokines, the activation of T cells and natural killer cells, followed by the release of the cytokines, rapid recruitment of circulating inflammatory cells, and encapsulation by fibrosis.

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**REFERENCES**


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